METHOD DEVOLOPMENT AND VALIDATION OF ANTINEOPLASTIC DRUG IN SOLID DOSAGE FORM USING HPLC METHOD

A dissertation submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI- 600 032.

In partial fulfillment of the requirements for the award of Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted

BY

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Under the guidance of

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CERTIFICATE

This is to certify that the dissertation entitled **"METHOD DEVOLOPMENT AND VALIDATION OF ANTINEOPLASTIC DRUG IN SOLID DOSAGE FORM USING HPLC METHOD"** submitted by **Ali Ebraheem Fadlallah Ebraheem** (Reg No: 261330951)in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under my guidance in Department of Pharmaceutical Analysis, Edayathangudy.G.S.Pillay College of Pharmacy, Nagapattinam.during the academic year 2014-2015.

Place: Nagapattinam

Prof.Dr.D.BabuAnanth,M.Pharm., Ph.D.,

Date:

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1.0. INTRODUCTION

1.1. The Importance Of Newer Analytical Method[1]

Drug analysis entails identification, characterization and determination of drugs in mixtures such as dosage forms and biological fluids. The number of drugs acquainted into the market has been increasing at an alarming rate. These drugs may be either new entity in the market or partial structural modifications of the existing drug. Newer analytical methods are developed for these drugs or drug combinations of the following reasons:

- The drug or drug combinations mayn‟t be official in any pharmacopoeia.
- Exploring literature may not reveal an analytical method for the drug or its combinations.
- Analytical methods may not be available for the drug combination due to the interference caused by excipients.
- Analytical methods for the quantification of drug or drug combination from biological fluid may not be available or usable.
- Analytical methods for a drug in combination with other drugs may not be available.

On the other hand, the existing procedure may,

- Require expensive instruments, the reagents, solvents etc.

- Involve any tedious extraction or separation steps, which may be quite time consuming.

- Not be rapid, reliable or sensitive.

The newly developed analytical methods[2] find their importance in various fields like

- Research institutions,

- Quality control department in industries,

- Approved testing laboratories,

- Bio-pharmaceutics and Bio-equivalence studies,

- Clinical pharmacokinetic studies,

- Drug-drug interaction studies and

- Toxicology studies.

1.2. Estimation of drugs in dosage forms[3]

The methods of estimation of drugs are divided into physical, chemical, and physico-chemical and biological ones. Among these physico-chemical and physical methods are widely used. Physical methods of analysis involve the study of the physical properties of a substance. They include determination of the solubility, transparency or degree of turbidity, color, density or specific gravity (for liquids), moisture content and melting, freezing and boiling points. Physico chemical methods are employed to study the physical phenomenon that occurs as a result of chemical reactions.

Among the physicochemical methods the most important are optical (refractometry, polarometry, emission and the fluorescence methods of analysis,

photometry including photo colorimetry and spectrofluorimetry covering UV-Visible and I.R. regions, Nephelometry and turbidimetry), Electro chemical (potentiometry, colorimetry, amperometry, and polarography) and chromatographic methods like

(column, paper, thin layer, gas liquid, high performance liquid) etc.

Method involving nuclear reactions such as nuclear magnetic resonance

(N.M.R) and paramagnetic resonance (P.M.R) are becoming more and more popular. The combination of liquid chromatography with mass spectroscopy is one of the most powerful tools available.

The chemical methods include the gravimetric and volumetric procedures, which are based on complex formation, acid-base reactions, precipitations and red-ox reactions, titration in non-aqueous media and complexometric titrations are various types which are widely used in pharmaceutical analysis.

Analytical methods for the drugs in individual or combined dosage forms includes

a. **Classical separation or wet analysis (Non-instrumental)**

b. **Instrumental method of analysis**

- Spectral methods
- Electro analytical methods
- Chromatographic methods

Classical separation or wet analysis (Non-instrumental)

These processes subject the component of interest to classical separation techniques like extraction or isolation, then with a suitable estimation procedure namely gravimetry and volumetry etc.

Instrumental method of analysis

By this process, the component of interest is separated and or analysed by using the following techniques

i. Spectral Methods

The spectral techniques are used to measure electromagnetic radiation, which is either absorbed or emitted by the sample. E.g. UV-Visible spectroscopy, IR-spectroscopy, NMR & ESR-spectroscopy, flame photometry, flourimetry, atomic absorption spectroscopy and etc.

ii. Electro Analytical Methods

Electro analytical methods involve the measurement of current voltage or resistance as a property of concentration of the component in

olution mixture, eg. Potentiometry, conductometry and amperometry etc.

iii. Chromatographic Methods

Chromatography is a separation technique that is based on differing affinities of a mixture of solutes between at least two phases. The result is a physical separation of the mixture into its various components. The affinities or interactions can be classified in terms of a solute adhering to the surface of a polar solid (adsorption), a solute dissolving in a liquid (partition), and a solute passing through or impeded by a porous substance based on its molecular size (exclusion).

1.3. HPLC

The High Pressure or Performance Liquid Chromatography (HPLC) is a type of chromatography in which separation occurs between a pressurised liquid as mobile phase and a stationary phase contained in a column, one end of which is attached to source of pressure.[5,6]

The technique of High Performance Liquid Chromatography is so called because of its improvement performance when compared to classical column chromatography. It is also called as High pressure liquid chromatography since high pressure is used when compared to classical column chromatography.

te of distribution of drugs between stationary and mobile phase is controlled by diffusion process, if diffusion is minimized, a faster and effective separation can be achieved.

With HPLC, a pump (rather than gravity) provides the higher pressure required to propel the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows for a better separation on columns of shorter length when compared to ordinary column chromatography.

A liquid chromatography[7] consists of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample in to mobile phase, a chromatographic column, a detector, and a data collection device such as computer, integrator or recorder. Short, small-bore columns containing densely packed particles of stationary phase provide for rapid exchange of compounds between the mobile phase and stationary phases. In addition to receiving and reporting detector output, computers are used to control chromatographic settings and operations.

1.4. Principle of separation[8]

The principle of separation in normal phase mode and reverse phase mode is **adsorption**. When a mixture of components are introduced into a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the stationary phase travels slower. The component which has less affinity towards the stationary phase travels faster. Since no two components have the same affinity towards the stationary phase,the components are separated.

1.5. Types of HPLC techniques

i) Based on modes of chromatography

(a) Normal Phase chromatography

(b) Reverse Phase chromatography

ii) Based on principle of separation

(a) Adsorption chromatography

(b) Ion exchange chromatography

(c) Ion pair chromatography

(d) Size exclusion chromatography

(e) Affinity chromatography

(f) Chiral phase chromatography

iii) Based on elution technique

(a) Isocratic separation

(b) Gradient separation

iv) Based on the scale of operation

(a) Analytical HPLC

(b) Preparative HPLC

v) Based on the type of analysis

(a) Qualitative analysis

(b) Quantitative analysis

i) Based on Mode of Chromatography

a) Normal phase mode chromatography

In normal phase mode, the stationary phase (e.g. silica gel) is polar in nature and the mobile phase is non-polar. In this technique, non-polar compounds travel faster and are eluted first. This is because less affinity between solute and stationary phase. Polar compounds are retained for longer time in the column because more affinity towards stationary phase and takes more time to be eluted from the column.

This is not advantageous in pharmaceutical applications since most of the drug molecules are polar in nature and takes longer time to be eluted and detected. Hence this technique is not widely used in pharmacy.

b) Reverse phase mode chromatography

In reverse phase technique, a non-polar stationary phase is used. The mobile phase is polar in nature. Hence polar components get eluted first and non-polar compounds are retained for a longer time. Since most of the drugs and pharmaceuticals are polar in nature, they are not retained for a longer time and eluted faster, which is advantageous. Different columns are used are ODS (Octadecylsilane) or C18, C8, C4, etc.

1.2. LITERATURE REVIEW

1.3. Literature review of drug

Literature survey was carried out to enumerate the reported analytical methods for the selected drugs in individually or combination with other drugs.

- **Mahmood Ahmad *et al.,*** [12] validated and reported a rapid, sensitive and reproducible high performance liquid

  chromatographic (HPLC) method in plasma for simultaneous quantification of 5-fluorouracil, Adriamycin and

  Cyclophosphamide (FAC) using C18 column with mobile phase consisting of 0.05 M disodium hydrogen phosphate and acetonitrile (65:35 v/v) containing 0.5 mL/L triethylamine (pH 3.7).The flow rate was 0.650 mL/min. UV detection of FAC was set at 266, 254 and 198 nm respectively. Total run time was 15 min and retention times for 5-fluorouracil, cyclophosphamide and adriamycin were 4.1, 7.7 and 10.9 min respectively.

- **Isarita Martins *et al.,*** [13] reported Simultaneous determination of Cyclophosphamide and Ifosfamide in Plasma using SPE-

  HPLC-UV method. The assay was performed by HPLC-UV, with a C18 column (5µm, 150 x 4 mm) and detection in 195 nm. The mobile phase was constituted by phosphate buffer 10 mM pH 6.0 : acetonitrile (77.25 : 22.75), with a flow of 1mL/min.

- **Rodney R. Larson *et al.,*[14]** reported HPLC Method development for simultaneous analysis of five antineoplastic agents using

  Waters Symmetry C8 column with mobile phase consisting of

  22.75 percent acetonitrile in water buffered to a pH of 6.0. The detection was performed by UV detector at 195nm.

- **Abu M. Rustum *et al.,*[15]** had developed a RP-HPLC method for the determination of Cyclophosphamide in blood and plasma

  using a short column packed with 5-µm reversed-phase octadecylsilane (ODS) with an isocratic elution of 5 m*M* potassium phosphate (pH 6.80) & acetonitrile in the ratio 80:20 v/v at a flow-rate of 1.0 ml/min. The detection was performed by UV detector at 195nm.

- **Terry T. Kensler *et al.,*[16]** reported High-performance liquid chromatographic analysis of cyclophosphamide in the presence

  of its hydrolysis products. The drug was quantified using a UV detector at a low wavelength. A single band was observed for the intact drug, which was well separated from its hydrolysis product(s).

- **Nagulu Malothu *et al.,*[17]** developed and reported a rapid and simple HPLC method for the determination of cyclophosphamide

  in human serum using 250×4.6 mm ODS column with mobile phase consisting of water and acetonitrile (70:30) at a flow rate of 1.2ml/min. Para aminoacetophenone was used as internal

standard. The detection was performed by UV detector at

197nm.

- **LI Su-hua *et al.,*** [18] reported HPLC method development for determining the concentration of Cyclophosphamide in the

  plasma of mice using diamonsil C18 column, with the detective UV wavelength of 200nm.Acetonitrile and water with the ratio of 18 : 82 (V/V) was used as mobile phase at a flow rate of 0.5ml/min.

- **Respaud.R *et al.,*** [19] reported Fast Analysis of Chemotherapy Drugs with the Agilent Pursuit XRs Ultra Diphenyl Column.

  Using an HPLC system with Agilent Polaris and Agilent Pursuit columns, researchers at the Hôpital Européen Georges

  Pompidou validated a method that differentiated cancer drugs in less than three minutes. This is a major step in developing an analytical verification procedure to improve the quality of drug delivery for cancer patients, without compromising patient experience.

- **Zhuorong Li *et al.,*** [20] reported Nitrobenzo-cyclophosphamides

  As potential prodrugs for bioreductive activation using Eschericia coli nitroreductase. Series of four benzo cyclophosphamide analogues were designed and

  synthesized incorporating a strategically placed nitro group in a position para to the benzylic carbon for reductive activation.

2.2. DRUG PROFILE

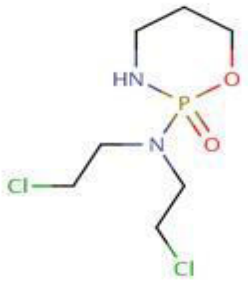
2.2.1. CYCLOPHOSPHAMIDE (CP)[46]

Description

Cyclophosphamide is a synthetic alkylating agent chemically related to the nitrogen mustards with antineoplastic and immunosuppressive activities. It is a prodrug that requires activation by the cytochrome P450 enzyme system to form its pharmacologically active metabolite 4-hydroxyl cyclo-phosphamide. The metabolites are genotoxic due to their ability to cross-link DNA and thereby cause DNA damages.

Chemical structure

(2-[bis(2chloroethyl)amino]tetrahydro2H1,3,2oxazaphosphorine2oxide)



Generic name: Cyclophosphamide 50mg

Brand name: Cytoxan 50mg

Preparations: Powder for intravenous injection: 100, 200, 500, 1000, and 2000 mg. Tablets: 25, 50 mg.

Physical and chemical properties

Basic physical and chemical properties of CP are summarized below

Molecular formula: C7H15Cl2N2O2P

Molecular weight: 261.09

Physical properties: Odourless, fine white crystalline powder

Melting point: 49.5 – 53 ° C

Boiling Point: 336° C

Density: 1.479 g/cm3

Solubility: sparingly soluble in water, methanol, Acetonitrile and slightly soluble in ethanol

Partition coefficient: 0.63

pKa: 4.5-6.5

Stability: Hydrolysis occurs at temperatures above 30 °C, with removal of chlorine atoms. Sensitive to oxidation, moisture and light.

Mechanism of action of CP

First CP is converted by the liver into two chemicals acrolein and phosphoramide. Acrolein and phosphoramides are the active compounds that prevent cell division by cross linking DNA strands and decreasing DNA synthesis.

Pharmacokinetics

Absorption, distribution and elimination of CP

CP is well absorbed orally, with peak concentrations occurring after 1-3 hours and has bioavailability of 85-100%. The drug is rapidly absorbed from the blood after IV injection.

CP is distributed with a volume of distribution of 30-50 L, which approximates to the total body water. Penetration of CP and its metabolites into body fluids is limited. Metabolites like

CarboxyCP and DicarboxyCP were not found in cerebrospinal fluid whereas PM and CP could be detected.

CP and its metabolites are eliminated by urine in 24 hours after the start of treatment. The major function of CP in the body is eliminated by hepatic metabolism, but small fraction is eliminated by renal excretion of unchanged drug in urine.

Small fraction of CP dose is eliminated via faeces and expired air. The elimination half-life of CP ranges between 5-9 hours over a large concentration range. The plasma half-life of CP is approximately 5 h in human during the first day after the dose.

Cyclophosphamide use

CP has been widely in different disease treatments

) Breast cancer

a) Ovarian cancer

b) Prostate cancer

c) Lung cancer

 Lupus erythematosus

 Rheumatoid arthritis

 Multiple Sclerosis

 Idiopathic pulmonary fibrosis

 Wegener granulomatosis

- Hodgkin’s Lymphoma
- Thrombocytopenic purpura
- Polyarteritisnodosa
- Anaemia

Side effects of Cyclophosphamide[47]

It includes-

- Hair loss
- Vomiting
- Diarrhoea
- Mouth sores
- Weight loss
- Sterility
- Jaundice

Adverse Effects[48]

1.9.1. black, tarry stools

1.9.2. red urine

1.9.3. unusual bruising or bleeding

1.9.4. unusual tiredness or weakness

1.9.5. sore throat, cough, fever, or other signs of infection

- swelling in the legs, ankles, or feet

- chest pain

Drug Interactions[48]

- The rate of metabolism and the leukopenic activity of cyclophosphamide reportedly are increased by chronic administration of high doses of phenobarbital.

- Cyclophosphamide treatment causes a marked and persistent inhibition of cholinesterase activity and potentiates the effect of succinylcholine chloride.

- Allopurinol (Zyloprim) enhances the ability of cyclophosphamide to reduce production of blood cells from the bone marrow.

- Increases the occurrence of heart failure that is caused by doxorubicin (Adriamycin),

- Increases the action of blood thinners such as warfarin (Coumadin), and decreases the effectiveness of quinolone antibiotics (Cipro).

Dosage[48]

- The usual initial dose of cytoxan for adults and children is 40-50 mg/kg administered intravenously over 2-5 days in divided doses. This dose may repeated at 2-4 week intervals.

- The usual oral dose is 1-5 mg/kg daily.

3.0. AIM AND OBJECTIVE

The number of drugs and drug formulations introduced into the market has been increasing at an alarming rate. These drugs or formulations may be either new entities in the market or partial structural modifications of the existing drug or novel dosage forms.

Most of the pharmaceutical industries rely upon quantitative chemical analysis to ensure that the raw material used and the final product thus obtained meets certain specifications and to determine how much of each component present in the final product.

Standard analytical procedures for these drugs or formulations may not be available to develop newer analytical methods which are accurate, precise, specific, linear, simple and rapid.

Moreover in the early part of the century only colorimetric and spectrophotometric methods were used for drug analysis due to reasons of economy and easy availability. These methods, however, are used to lesser extent today because they lack specificity, sensitivity and accuracy. The modern method of choice of assays is

High – Pressure Liquid Chromatography (HPLC) which is a powerful and rugged method. It is also extremely precise, accurate, sensitive, specific, linear and rapid.

The objective of the present research is to develop and validate a precise, accurate and robust method for the estimation of

Cyclophosphamide from tablet dosage form and the extensive literature survey carried out revealed that several methods have been reported for simultaneous

estimation of Cyclophosphamide i.e., in combination with other drugs. However, there is no method reported for individual estimation in solid dosage form.

3.0. PLAN OF WORK

The plan of the work for the aimed study was designed as follows:

1.4. Method development and optimization of chromatographic conditions

Selection of wavelength

Selection of initial separation conditions

Nature of the stationary phase

Nature of the mobile phase (pH, peak modifier, solvent strength, ratio and flow rate)

Sensitivity

1.5. Validation of developed HPLC method (Acc. to ICH guidelines)

The method developed were also proposed to validate using the

various validation performance parameters such as,

- Selectivity/specificity
- Linearity and range

- Accuracy
- Precision (repeatability and reproducibility)
- Limit of detection (LOD)/ Limit of quantification (LOQ)
- Robustness
- System suitability

1.0. MATERIALS REQUIRED

1.1. Instruments used

Table 1: Instruments Used During the Method Development

S.No.	Name	Model	Make	Software
1.	Analytical Balance	MS205DU	Mettler Toledo	-
2.	Micro Balance	MX5	Mettler Toledo	-
3.	pH Meter	LP139SA	POLMON	-
4.	Centrifuge	R-8CBL	REMI	-
5.	Ultra Sonicator	-	S V Scientific	-
6.	Oven	-	S V Scientific	-
7.	UV Spectrophotometer	Lambda-25	Perkin Elmer	UV Winlab
8.	HPLC	Agilent-1200 infinity series	Agilent	Open lab control panel E Z chrome
9.	UV-Visible / PDA Detector	PDA-1200 infinity series	Agilent	-
10.	Water Purifier	MilliQ	Millipore	-

5.2. Chemicals used

Table 2: Chemicals/Reagents Used During the Method Development

S.NO.	Name of the Chemical/Reagent	Make	Grade
1.	Sodium Hydroxide	SD fine chemicals Ltd	AG
2.	Hydrochloric Acid	SD fine chemicals Ltd	AG
3.	Hydrogen Peroxide	SD fine chemicals Ltd	AG
4.	Acetonitrile	Merck	HPLC
5.	Water	MilliQ	HPLC
6.	Methanol	Merck	AG
7.	0.45µm Nylon Filter	Millipore	AG

For the determination of Cyclophosphamide in tablet dosage form by RP-HPLC method the following Standard drug was used as a reference standard.

S. No.	Name	Specification purity %	Gift sample
1	Cyclophosphamide	99.7	Celon Labs.

For the determination of Cyclophosphamide in tablet dosage form by RP-

HPLC method the following marketed formulation was used.

Brand name	Content	Manufacturing company
Cytoxan	Cyclophosphamide(50mg)	Celon Labs, India

1.6. EXPERIMENTAL INVESTIGATION

1.7. Method development of cyclophosphamide

A new RP-HPLC method was developed for the determination of Cyclophosphamide. The HPLC method was then validated to indicate that the analytical procedure used is suitable for intended use by using various parameters like specificity, linearity, accuracy, precision, robustness, and system suitability.

6.1.1. Selection of Initial Conditions for Method Development

a. Determination of Solubility of Drug

Table 3: Solubility Analysis of drug.

Solvent	Solubility
Water	Sparingly soluble
Methanol	
Acetonitrile	
Ethanol	Slightly soluble

b. Determination of Absorption Maxima by PDA detector

Preparation of Diluent

Water: Acetonitrile (50:50)

Procedure

Accurately weighed and transferred about 50 mg of Cyclophosphamide into a 50 ml volumetric flask, added to it about 75 ml of diluent and sonicated to dissolve and diluted up to the mark with diluent and mixed well. Further diluted 5 ml of the above solution to 50 ml with diluent and mixed. (Concentration of Cyclophosphamide is about 0.1mg/ml). The PDA spectrum was shown in figure 2.

c. Choice of instrument: High performance liquid chromatography.

Preparation of Cyclophosphamide stock solution

50mg of Cyclophosphamide was weighed and taken into clean 50mL dry volumetric flask and added small quantity of diluent for solubilising the drug and sonicated for about 10min and finally make up the solution with diluent.

Preparation of working solution

Form the stock solution transferred 5 ml of the solution into a clean and dry 50mL volumetric flask and added small quantity of diluent and sonicated for about 10min and finally made up the volume with diluent.

6.1.2 Method development trials

Trail-1

Mobile phase preparation

The mobile phase was prepared by mixing water and acetonitrile in the ratio of 50:50. Then it was filtered and degassed.

Chromatographic conditions

Standard solution of Cyclophosphamide was loaded in the vial, injected and run for 15min. The HPLC parameters were set in the

method as follows. The trial plot was shown in figure 3.

- Column : C18 100×4.6mm; 3.5µm
- Column temperature : 25^0C
- Flow rate : 0.5mL/min
- Injection volume : 20µL
- Wave length : 195nm

Trail-2

Mobile phase preparation

The mobile phase was prepared by mixing water and acetonitrile in the ratio of 60:40. Then it was filtered and degassed. The trial plot was shown in figure 4.

Chromatographic conditions

- Column : C18 100×4.6mm; 3.5µm
- Column temperature : 25^0C
- Flow rate : 0.5mL/min
- Injection volume : 20µL
- Wave length : 195nm

Trail-3

Mobile phase preparation

The mobile phase was prepared by mixing water and acetonitrile in the ratio of 75:35. Then it was filtered and degassed. The trial plot was shown in figure 5.

Chromatographic conditions

- Column : C18 100×4.6mm; 3.5µm
- Column temperature : 25^0C
- Flow rate : 0.5mL/min
- Injection volume : 20µL
- Wave length : 195nm

Conclusion: After trails it was concluded that the mobile phase which is suitable was Water: acetonitrile (70:30) and injection volume is 20 µL.

Trail-4 (Optimized)

Preparation of mobile phase

Mix water and Acetonitrile in ratio of 70:30 (v/v) and degas and filter through 0.45 micron membrane filter.

Diluent: Mobile phase itself is taken as diluent.

Chromatographic conditions

The HPLC parameters were set in the method as per table 4.The optimized plot was shown in figure 6

Table 4: Chromatographic conditions

Parameters	Description
Column	Agilent Zorbax Eclipse plus 100 x 4.6mm;3.5µ.
Column temperature	25^0C
Mobile phase	Water : Acetonitrile (70:30)
Flow rate	0.5mL/min
Auto Sampler temperature	25^0C
Injection volume	20µL
Detector wavelength	195nm
Run time	15min

6.2 Analytical method validation

Validation of an analytical method is a process to establish by laboratory studies that the performance characteristic of the method meets the requirements for the intended analytical application. Performance characteristics are expressed in terms of analytical parameters. The proposed method was validated according to ICH guidelines.**6.2.1 Validation parameters**

i. Specificity/forced degradation studies

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures.

Cyclophosphamide Identification: Solutions of standard and Sample were prepared as per test method and injected into the chromatographic system. The specificity plots were shown in figure 7 and 8.

Acceptance criteria: Chromatogram of Standard and sample should be identical with near/same Retention time.

Blank interference: A study to establish the interference, blank detection was conducted. Mobile phase was injected as per the test method. . The specificity plot was shown in figure 7.

Acceptance criteria: Chromatogram of blank did not show any peak at the retention time of analyte peak.

Forced degradation studies

Degradation studies were carried out as per ICH guidelines. The objective of the study was to find out the degradation products, which in turn help in the establishment of degradation pathways and the intrinsic stability of drug molecule. In order to check the selectivity

the proposed method, degradation studies were carried out by using acidic, basic, oxidative, photo and thermal conditions.

Intentional degradation was attempted to stress conditions of acidic (0.1N HCl), basic/alkali (0.1N NaOH), oxidative degradation

(5%H2O2) and thermal treatment (heated at 80^0c) to evaluate the ability of the proposed method to separate cyclophosphamide from its degradation products.

A. Acidic degradation

Forced degradation in acidic media was performed by taking weighed powdered sample equivalent to 50mg, in to 50mL volumetric flask. 10mL of 0.1N Hcl was added and refluxed at 70^0C/30min. Then 10mL of 0.1N NaOH was added and volume was made up to mark with diluent. 5 to 50mL of further dilution was made to get 0.1mg/mL of cyclophosphamide and inject the sample.

B. Alkali degradation

Forced degradation in alkali media was performed by taking weighed powdered sample equivalent to 50mg, in to 50mL volumetric flask. 10mL of 0.1N NaOH was added and refluxed at 70^0C/30min. Then 10mL of 0.1N Hcl was added and volume was made up to mark with diluent. 5 to 50mL of further dilution was made to get 0.1mg/mL of cyclophosphamide and inject the sample.

C. Oxidative degradation

Forced oxidative degradation was performed by taking weighed powdered sample equivalent to 50mg, in to 50mL volumetric flask. 10mL of 5% H2O2 was added and refluxed at 70^0C/30min. Then volume was made up to mark with diluent. 5 to 50mL of further dilution was made to get 0.1mg/mL of cyclophosphamide and inject the sample.

D. Thermal Degradation

Cyclophosphamide pure drug was exposed to a controlled temperature oven NMT 80ºc for half an hr. At sampling time accurately weighed powdered sample equivalent to 50mg, in to 50mL volumetric flask. Then volume was made up to mark with diluent. 5 to 50mL of further dilution was made to get 0.1mg/mL of cyclophosphamide and inject the sample.

ii. Linearity and Range

Preparation of solutions for linearity

From the stock solution transferred 3.5ml, 4.0 ml, 4.5 ml, 5.0 ml, 5.5 ml, 6.0 ml, of Cyclophosphamide each into a series of 50 ml volumetric flasks and add a few ml of diluent and sonicated for 15 min and later volume was made up with diluent. The final concentrations for linearity of Cyclophosphamide were 70-120µg/mL.

The obtained value is y= 20405x - 244.681, R^2 =0.999. The results were shown in figures 14-19 and tabulated in the table no: 7.

iii. Accuracy

Preparation of solutions for Accuracy

From the stock solution transferred 4.0, 5.0 and 6.0 ml of solutions into 3 separate 50 ml volumetric flasks and dilute each of the flasks to 50 ml with mobile phase to get the desired concentrations of 80, 100 &120 mcg/mL respectively.These solutions were injected in 3 replicates. The percentage recovery was calculated from the data obtained and the results were shown in figures 20-28 and tabulated in the table no: 8.

iv. Precision

The precision studies were studied using six replicate measurements at 0.1mg/mL. Statistical evaluation revealed that relative standard deviation of drug at different concentration for six injections was less than 2.0. The results were mentioned in the table no-9.Intermediate precision was established for the same analytical samples of concentration 0.1mg/mL. The typical variation was included like interday analysis. Statistical evaluation revealed that relative standard deviation of drug at different concentration for six injections was less than 2.0. The results were mentioned in the table no-10.

v. Limit of detection

50mg of cyclophosphamide was weighed and make up to 50mL with mobile phase. From the above solution different concentrations of solutions were prepared as stated above i.e like linearity and the solutions were run as per chromatographic conditions. Based on the standard deviation of response and slope the LOD value of drug was calculated.

vi. Limit of quantification

50mg of cyclophosphamide was weighed and make up to 50mL with mobile phase. From the above solution different concentrations of solutions were prepared as stated above i.e like linearity and the solutions were run as per chromatographic conditions. Based on the standard deviation of response and slope the LOQ value of drug was calculated.

vii. Robustness

The robustness of an analytical procedure was tested by measuring its capacity to remain unaffected by small, but deliberate variations in the method parameters and provides an indication of its reliability during the normal use. For this study flow, mobile-phase composition, column parameters were changed. The results were shown in figures 41-45 and tabulated in the table.no:11-13.

viii. System Suitability

50mg of cyclophosphamide was weighed and taken into clean volumetric flask and added small quantity of mobile phase for solubilising the drug and sonicated for about 10min and finally make up the solution with mobile phase Pipette out 5ml of above solution was transferred into 50ml volumetric flask and make up the volume with

mobile phase to obtain concentration of 0.1mg/mL. This Standard solution was injected and the system suitability parameters are calculated and tabulated in the table no: 14.

6.3 Assay for Marketed formulation

Preparation of standard solution

50mg of cyclophosphamide was weighed and taken into clean volumetric flask and added small quantity of mobile phase for solubilising the drug and sonicated for about 10min and finally make up the solution with mobile phase Pipette out 5ml of above solution was transferred into 50ml volumetric flask and make up the volume with mobile phase to obtain concentration of 0.1mg/mL.

Preparation of sample solution

Cytoxan tablets; claimed to contain 50mg of Cyclophosphamide was used in analysis. A total of 5 tablets were accurately weighed and powdered in a mortar. An amount equivalent to 50mg of Cyclophosphamide was taken and dissolved in some amount of diluent and sonicated for 20mins and made upto 50ml in

50ml volumetric flask. The solution was transferred through a Whatmann No.1 filter paper. Further 5ml of the above solution was pipetted out into a 50ml volumetric flask and the volume was made up to 50ml with diluent(mobile phase) to obtain a concentration of 0.1mg/mL.

Procedure

The prepared standard and sample solutions were injected in the HPLC by setting the optimized chromatographic conditions. The peak areas of standard and sample were determined. These values are

substituted in the following formula to get the percentage purity of the marketed formulation. The results were shown in figure 47 and tabulated in the table no: 15.

7.0. RESULTS AND DISCUSSION

7.1. Results for method development trials

Absorption maxima

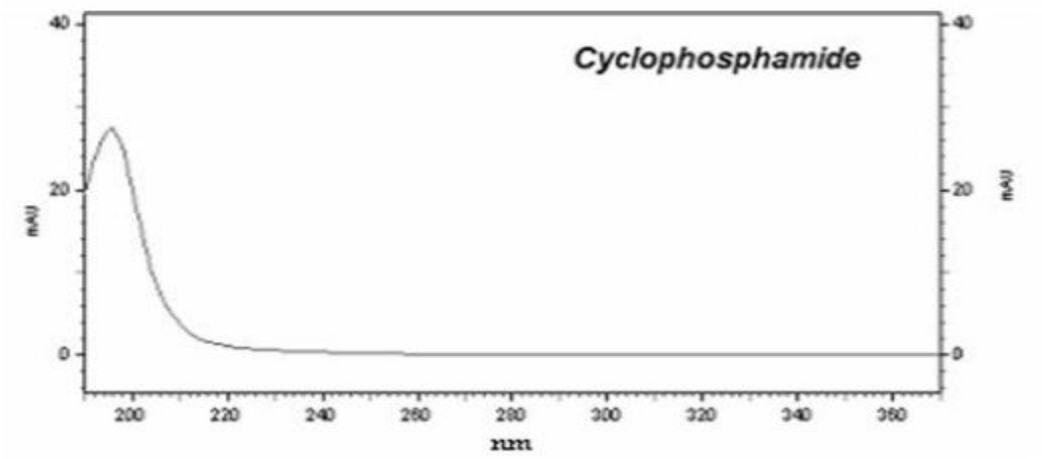


Figure 2: PDA spectrum of Cyclophosphamide

Trial no. 1

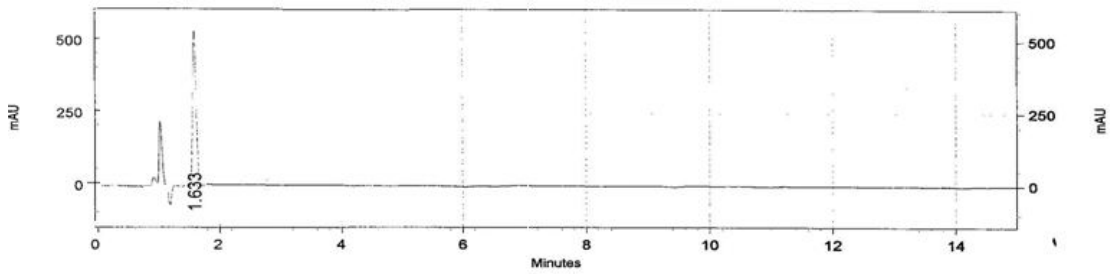


Figure 3: HPLC chromatogram of trial 1

Discussion

Peak was eluted early & Some Placebo peaks were appeared. so the mobile phase has been changed for next trail.

Trial no.2

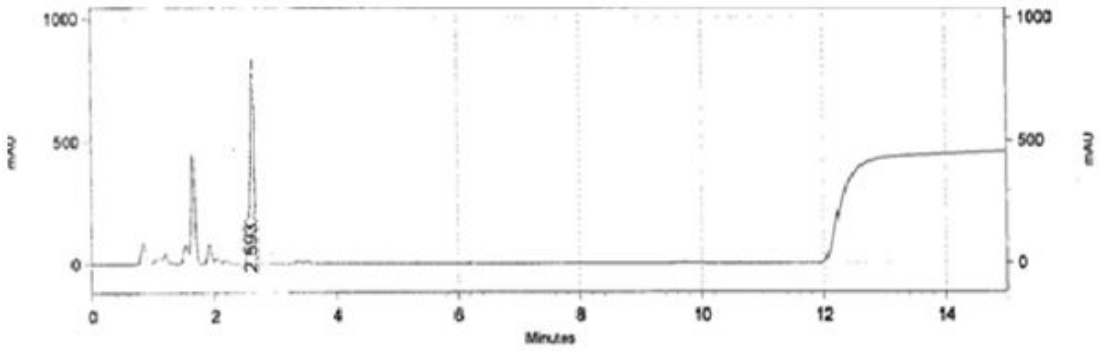


Figure 4: HPLC chromatogram of trial 2

Discussion

Placebo peaks were still appeared & Base line was not proper. so again gone for changing of mobile phase

Trial no. 3

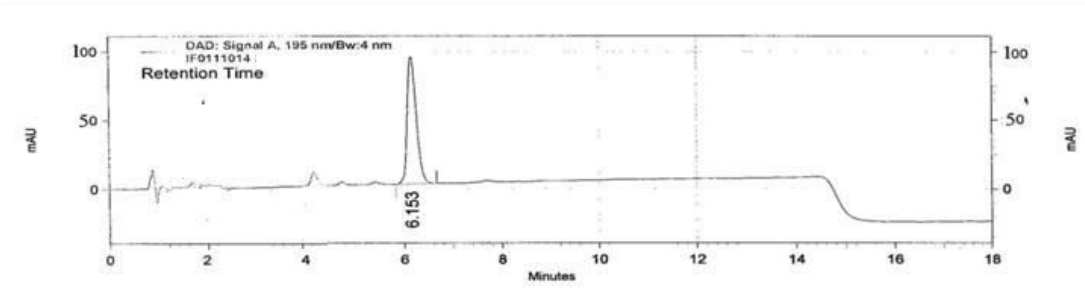


Figure 5: HPLC chromatogram of trial 3

Discussion

Placebo peaks were reduced but base line was not proper. so again gone for changing of mobile phase composition.

Trial no.4 (Optimized)

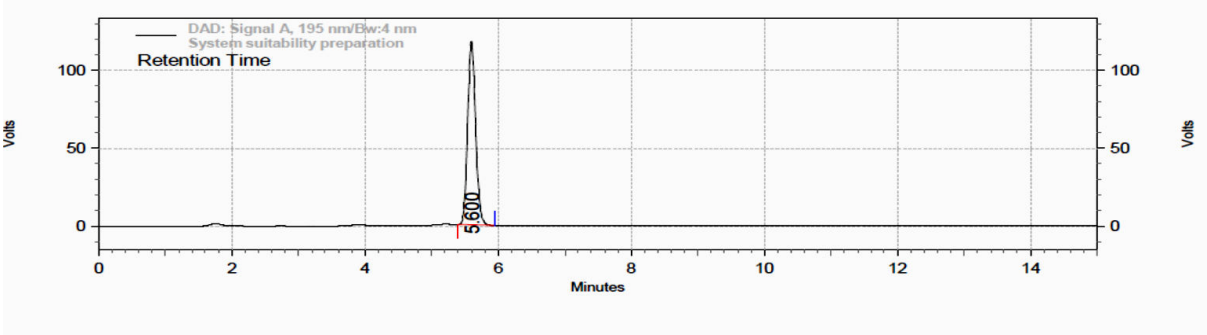


Figure 6: HPLC chromatogram of trial 4

Peak	Retention Time	Area	Area %	Theoretical plates(USP)	Asymmetry
Cyclophosphamide	5.600	2050439	100.00	10385	1.11

Discussion

The separation of analyte peak was achieved with good system suitability parameters. So these conditions are optimized for chromatographic run.

7.2. Results for method development

The optimized chromatographic conditions for the method development and validation of Cyclophosphamide is as follows

Optimized chromatographic conditions of Cyclophosphamide

Parameters	Description
Column	Agilent Zorbax Eclipse plus 100 x 4.6mm;3.5µ.
Column temperature	250C
Mobile phase	Water : Acetonitrile (70:30)
Flow rate	0.5mL/min
Sample temperature	25ºC

Discussion

The RP-HPLC method for Cyclophosphamide was optimized with the mobile phase consisting of Water & Acetonitrile (70:30). The detection was carried out at wavelength 195nm with a retention time of 5.6 min and peak asymmetry is 1.11.

7.3. Results for Analytical method validation

Validation of an analytical method is process to establish that the performance characteristics of the developed method meet the requirements of the intended analytical application. Typical analytical parameters used in assay validation are:

a) Specificity

b) Linearity and Range

c) Accuracy

d) Precision

- Repeatability
- Intermediate precision

e) Limit of Detection (LOD)

f) Limit of Quantification (LOQ)

g) Robustness

h) System suitability

a) SPECIFICITY

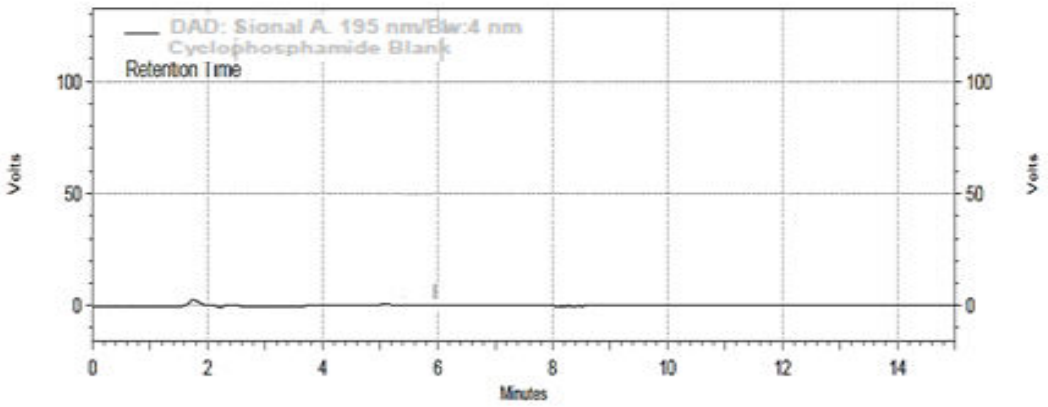


Figure 7 : HPLC Chromatogram of blank

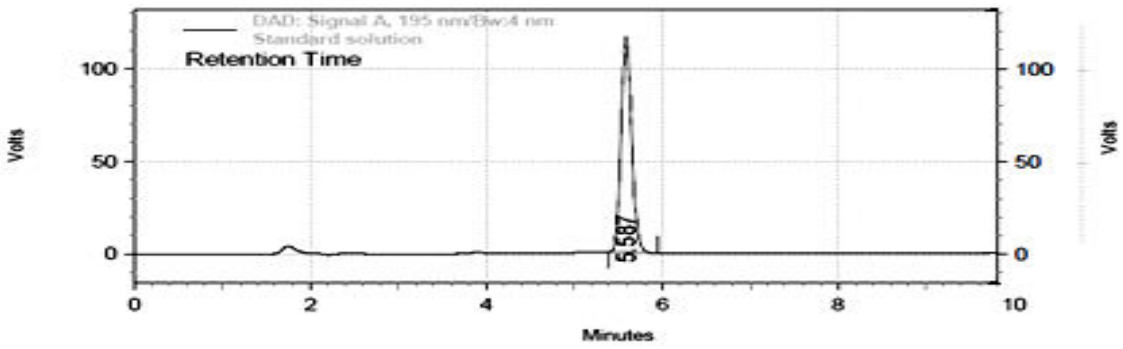


Figure 8 : HPLC chromatogram of standard

Peak results

Peak	*Cyclophosphamide*
Retention Time	*5.587*
Area	*2039272*
Area%	*100.00*
Theoretical plates (USP)	*10383*
Asymmetry	*1.12*

Degradation Studies

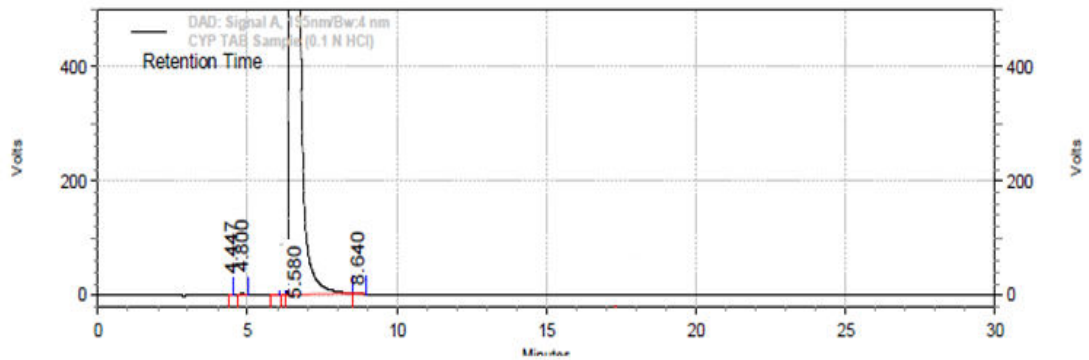


Figure 9: *HPLC chromatogram of acid degradation*

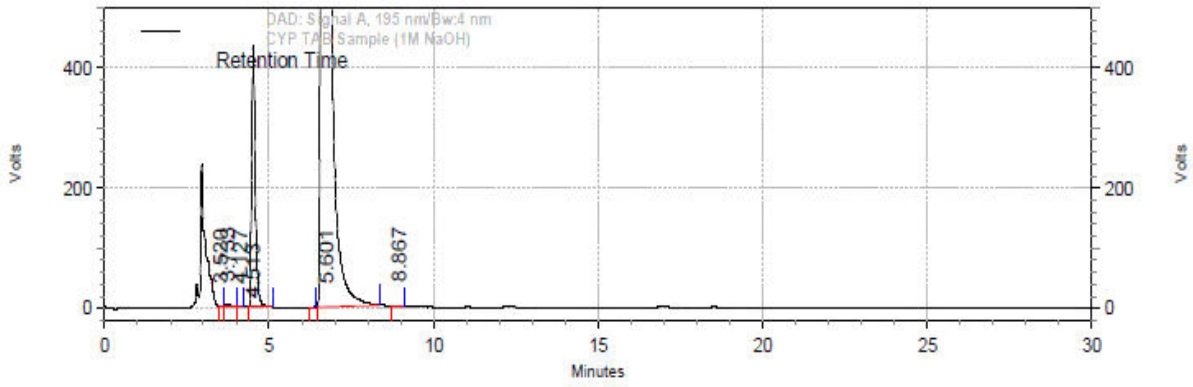


Figure 10 : *HPLC chromatogram of base degradation*

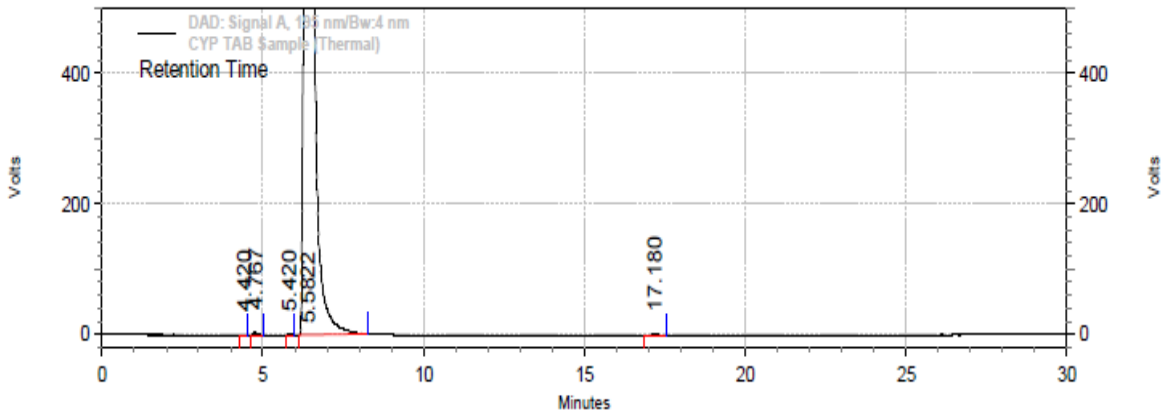


Figure 11: *HPLC chromatogram of heat degradation*

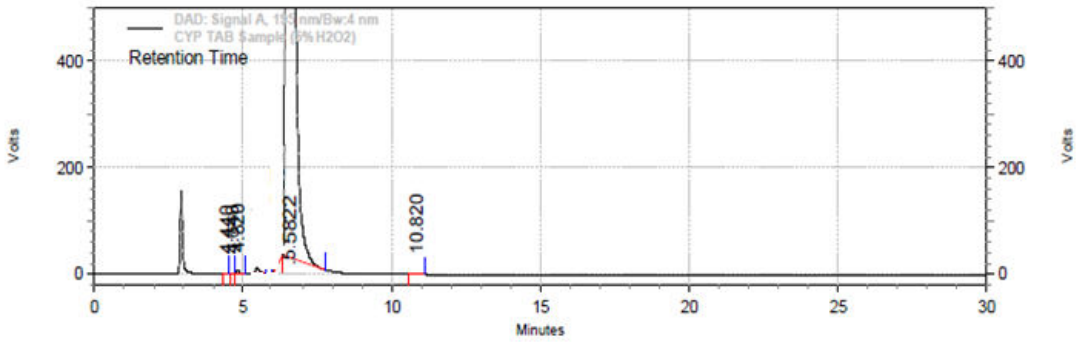


Figure 12: *HPLC chromatogram of peroxide degradation*

Table 6: Forced degradation results for Cyclophosphamide

Mechanism of degradation	**Observation**
Acid(0.1NHCl 30min reflux)	No interference at analyte peak
Base(1M NaOH 30 min reflux)	No interference at analyte peak
Heat at 50^0C for 1hr	No interference at analyte peak
Peroxide(5% H_2O_2 30 min	No interference at analyte peak

Discussion

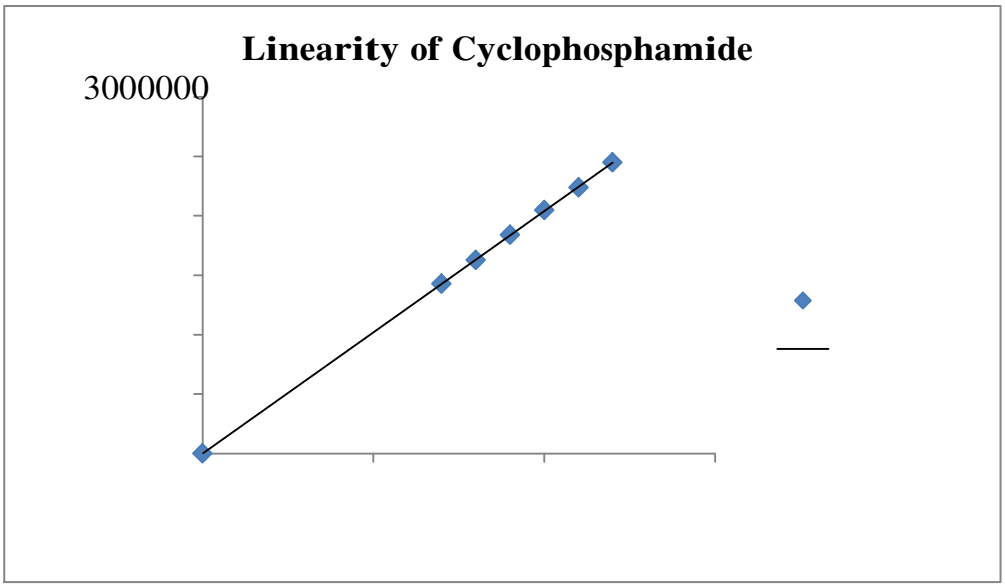
The HPLC chromatogram of blank does not contain any peaks, HPLC chromatogram of standard and sample containing Cyclophosphamide shows peaks at the same retention times. The HPLC chromatograms of degraded products show no interference at the analyte peaks.

b) LINEARITY & RANGE

The linearity of a method is its ability to obtain test results that are directly proportional to the sample concentration over a given range. The peak area and concentration were plotted to get a standard calibration curve. The correlation co-efficient and regression co-efficient were calculated.

Table 7: Linearity results of Cyclophosphamide

Sl no	Concentration mcg/ml.	Area
1	70	1427291
2	80	1627283
3	90	1840368
4	100	2048163
5	110	2239534
6	120	2447824
Slope		20407.33
y-intercept		-244.681
R^2 value		0.999



2500000

2000000

1500000

1000000

500000

y = 20405x R² = 0.999

Area

Linear (Area)

0 50 100 150

Concentration in µg/ml

Figure 13: Linearity plot of Cyclophosphamide

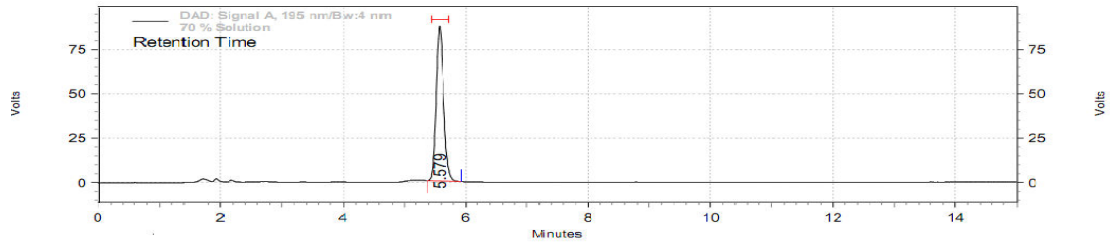


Figure 14: HPLC chromatogram of Cyclophosphamide linearity 70%

Peak results

Peak	Retention Time	Area	Theoretical plates	Asymmetry
Cyclophosphamide	5.579	1137297	9448	1.19

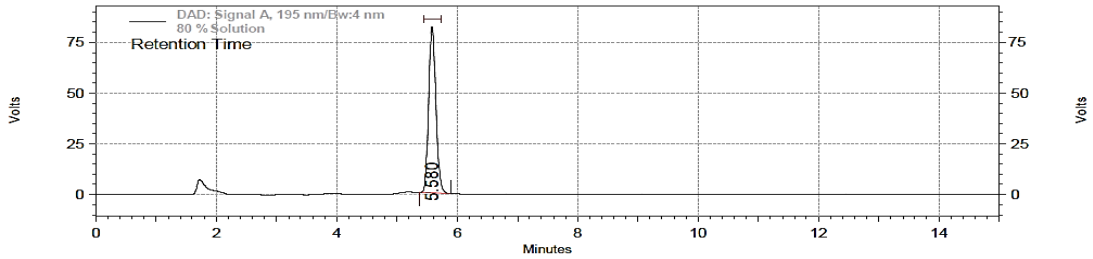


Figure 15: HPLC Chromatogram of Cyclophosphamide linearity 80%

Peak results

Peak	Retention Time	Area	Theoretical plates	Asymmetry
Cyclophosphamide	5.580	1427283	10311	1.16

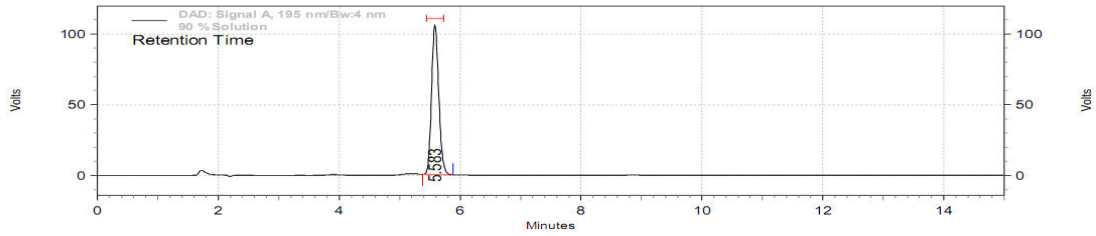


Figure 16: HPLC chromatogram of Cyclophosphamide linearity 90%

Peak results

Peak	Retention Time	Area	Theoretical plates	Asymmetry
Cyclophosphamide	5.583	1740368	10362	1.11

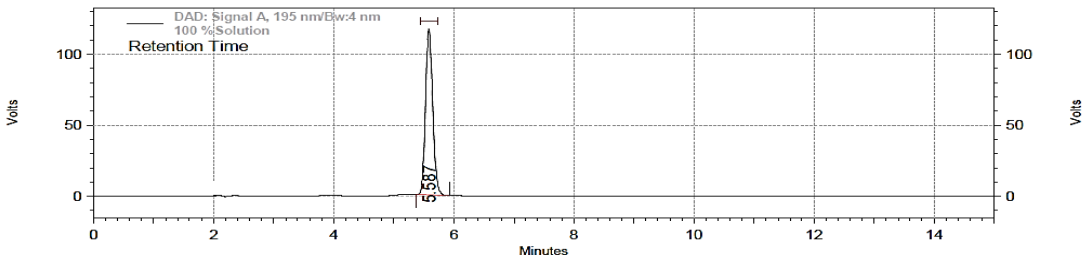


Figure 17: HPLC chromatogram of Cyclophosphamide linearity 100%

Peak results

Peak	Retention Time	Area	Theoretical plates	Asymmetry
Cyclophosphamide	5.587	2048163	10349	1.13

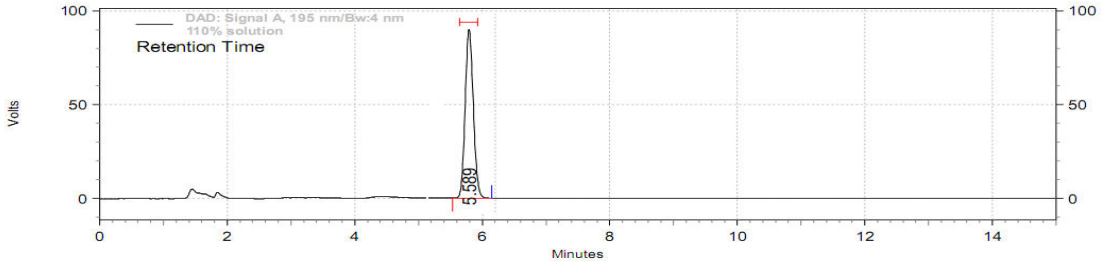


Figure 18: HPLC chromatogram of Cyclophosphamide linearity 110%

Peak results

Peak	Retention Time	Area	Theoretical plates (USP)	Asymmetry
Cyclophosphamide	5.589	2389534	8926	1.09

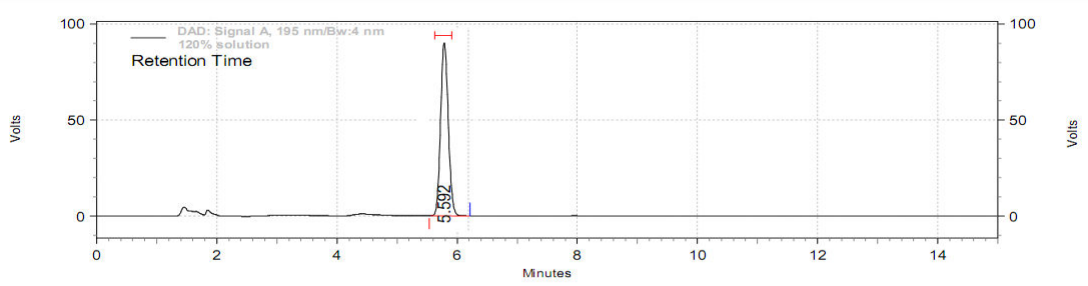


Figure 19: HPLC chromatogram of Cyclophosphamide linearity 120%

Peak results

Peak	Retention Time	Area	Theoretical plates (USP)	Asymmetry
Cyclophosphamide	5.592	2647825	8982	1.11

Discussion

Cyclophosphamide was found to be linear over the range of 70 to 120µg/mL. R^2 value for calibration plot of Cyclophosphamide was found to be 0.999

c) ACCURACY

The accuracy of a method is the closeness of the measured value to the true value for the sample. Accuracy is usually determined by recovery studies.

Table 8: Accuracy results for Cyclophosphamide

Spiked	Peak	Amount added	Amount found	% Mean
80% Prep-1	1457483	80.4	79.5	98.88
80% Prep-2	1469380	80.6	79.3	98.38
80% Prep-3	1465411	80.2	79.4	99.00
100% Prep-1	2035189	99.6	99.2	99.68
100% Prep-2	2045174	99.8	99.2	99.39
100% Prep-3	2053232	99.6	99.3	99.69
120% Prep-1	2654239	120	119.7	99.75
120% Prep-2	2665314	119.8	119.2	99.49
120% Prep-3	2674119	120	119.3	99.41
Mean				99.30
Standard deviation				0.46
%RSD				0.46

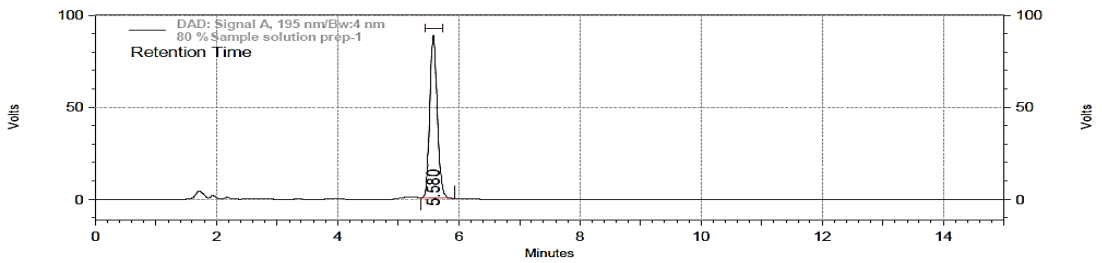


Figure 20: HPLC chromatogram of Cyclophosphamide for accuracy 80%

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.580	1457483	9730	1.15

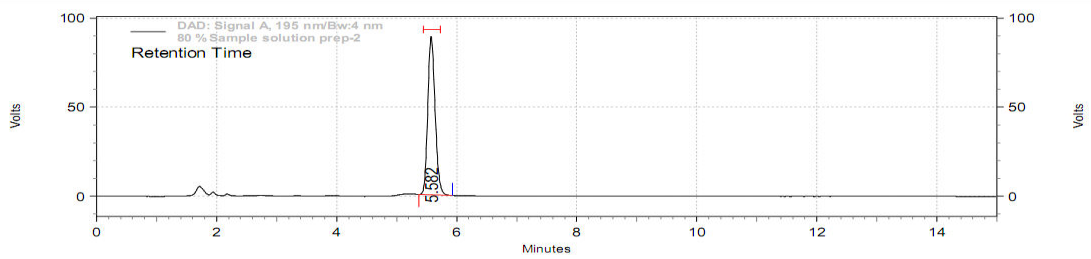


Figure 21: HPLC chromatogram of Cyclophosphamide for accuracy 80%

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.573	1469380	9782	1.16

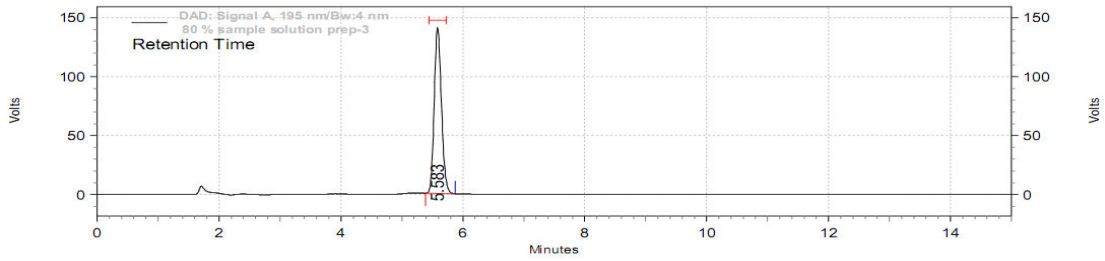


Figure 22: HPLC chromatogram of Cyclophosphamide for accuracy 80%

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.587	1465411	10457	1.13

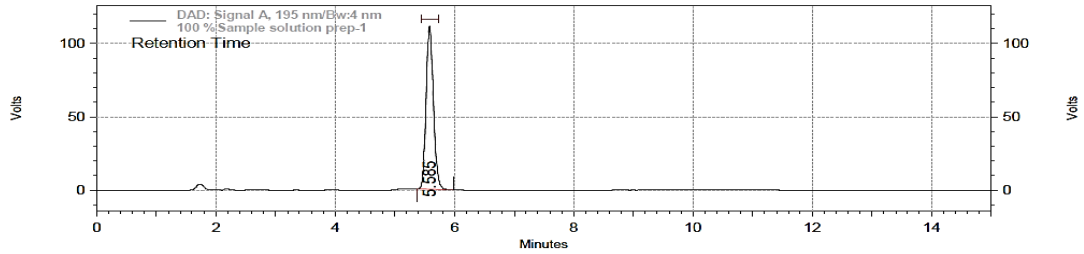


Figure 23: HPLC chromatogram of Cyclophosphamide for accuracy 100%

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamid	5.585	2035189	9761	1.17

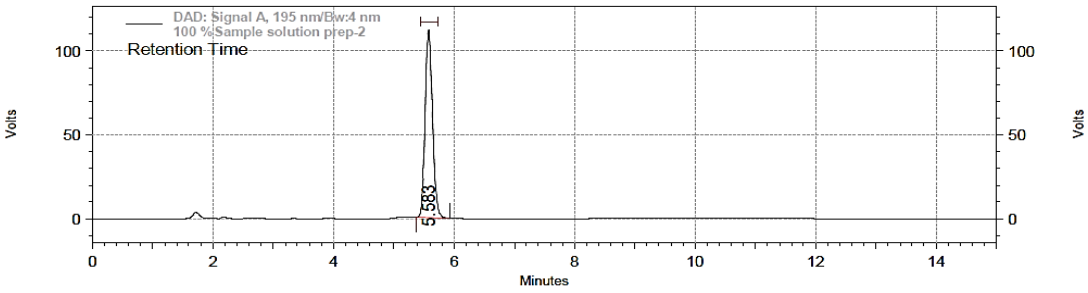


Figure 24: HPLC chromatogram of Cyclophosphamide for accuracy 100%

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.583	2045174	9893	1.13

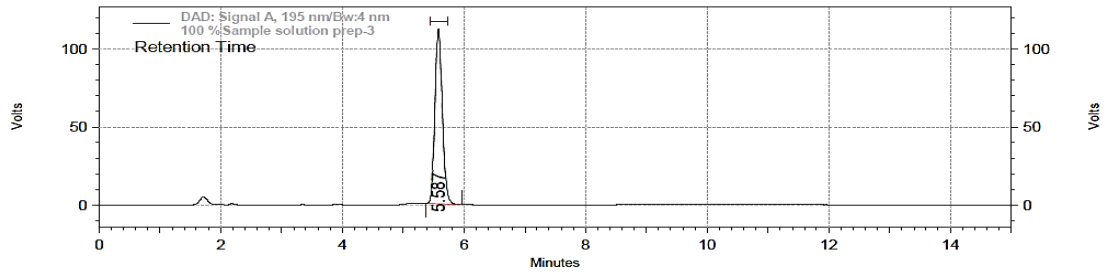


Figure 25: HPLC chromatogram of Cyclophosphamide for accuracy 100%

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.587	2053232	9950	1.14

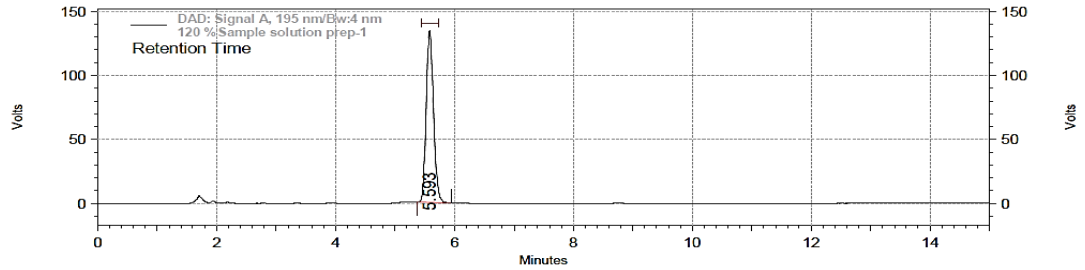


Figure 26: HPLC chromatogram of Cyclophosphamide for accuracy 120%

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.593	2654239	9783	1.18

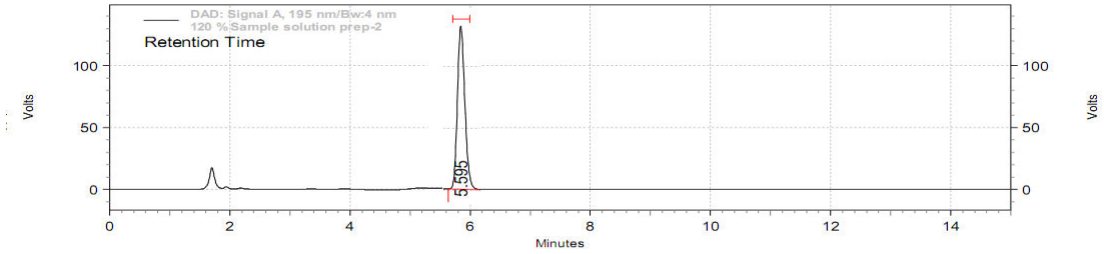


Figure 27: HPLC chromatogram of Cyclophosphamide for accuracy 120%

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.600	2665314	9604	1.16

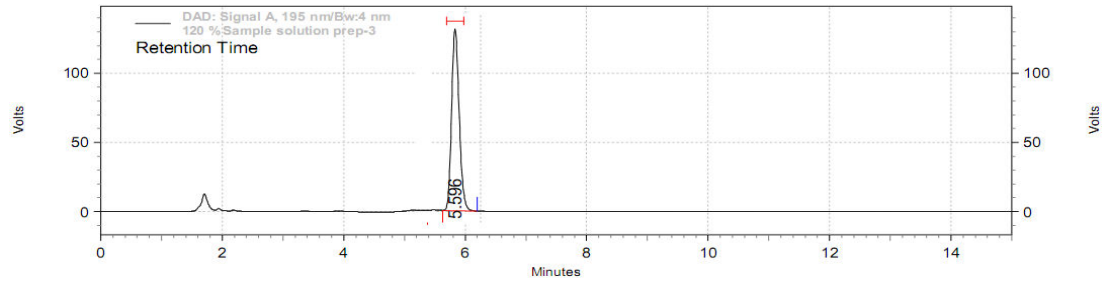


Figure 28: HPLC chromatogram of Cyclophosphamide for accuracy 120%

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.607	2674119	9542	1.15

Discussion

The percentage recovery of Cyclophosphamide was found to be 98.75%, 99.58% and 99.55% for accuracy 80%, 100% and 120% samples respectively. The %RSD of the samples was found to be less than 2.

d) PRECISION

Repeatability

The Repeatability studies were studied by six replicate measurements at 0.1mg/mL for Cyclophosphamide

Table 9: Precision results of Cyclophosphamide

Drug Conc.	S.No	Area
0.1mg/mL	Inj-1	2045164
	Inj-2	2044425
	Inj-3	2042132
	Inj-4	2053214
	Inj-5	2048155
	Inj-6	2051452
	Average	2047424
	STDEV	4298.60
	RSD	**0.21**

Discussion: The % RSD value indicates a good degree of precision within the specified range.

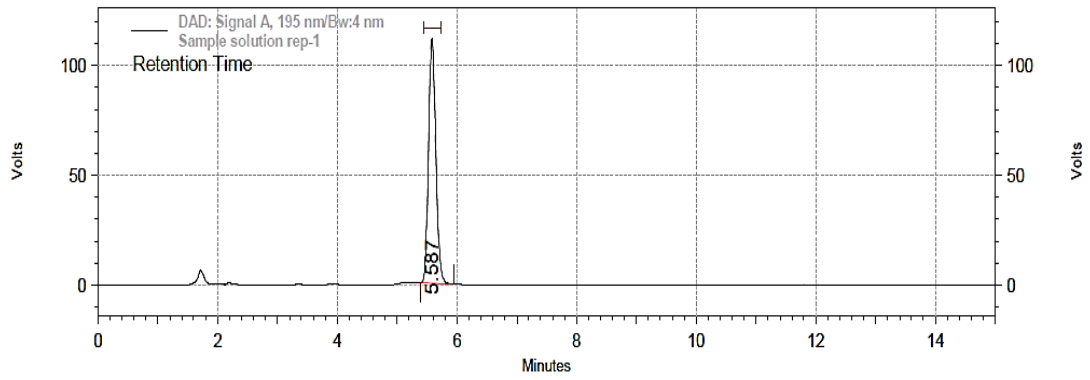


Figure 29: HPLC chromatogram of Cyclophosphamide for repeatability (inj-1)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.587	2045164	9975	1.18

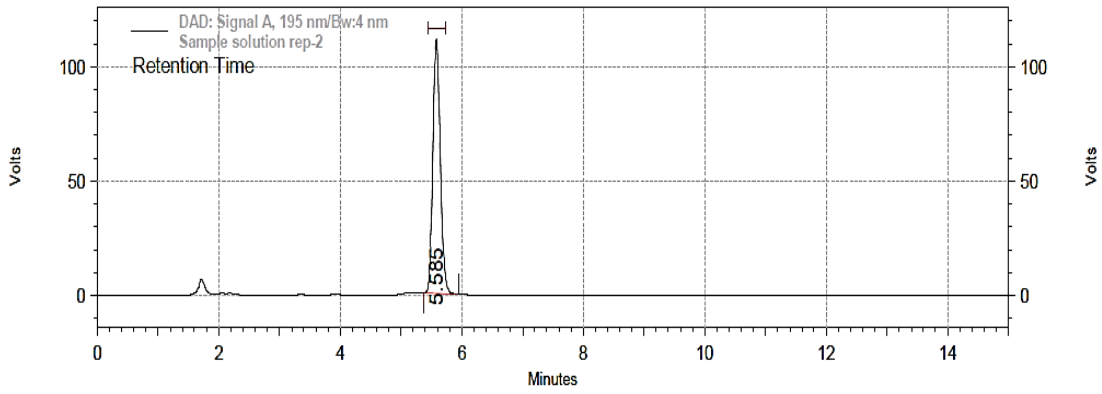


Figure 30: HPLC chromatogram of Cyclophosphamide for repeatability (inj-2)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.585	2044425	9866	1.16

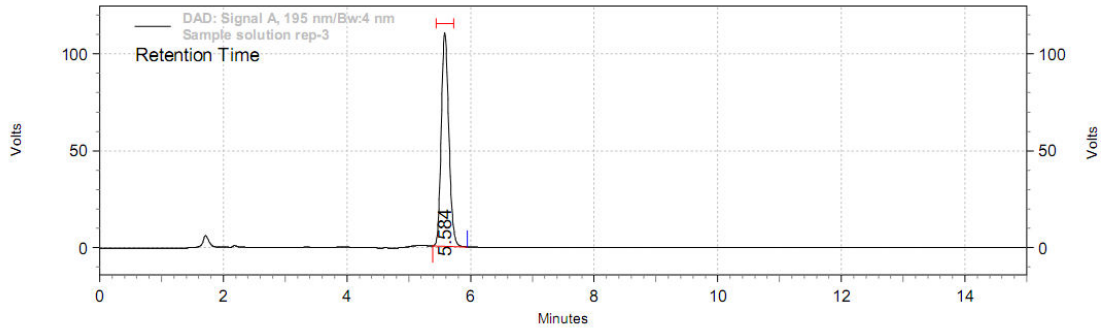


Figure 31: HPLC chromatogram of Cyclophosphamide for repeatability (inj-3)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.584	2042132	9829	1.12

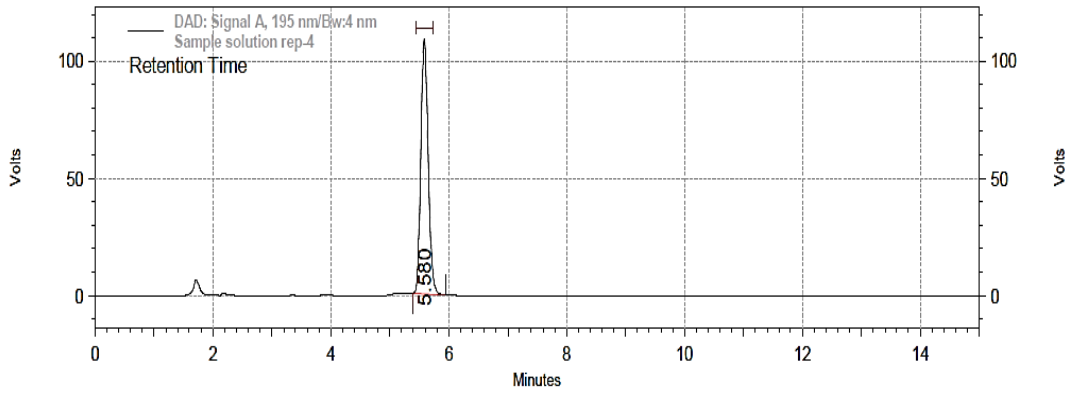


Figure 32: HPLC chromatogram of Cyclophosphamide for repeatability (inj-4)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.580	2053214	9587	1.13

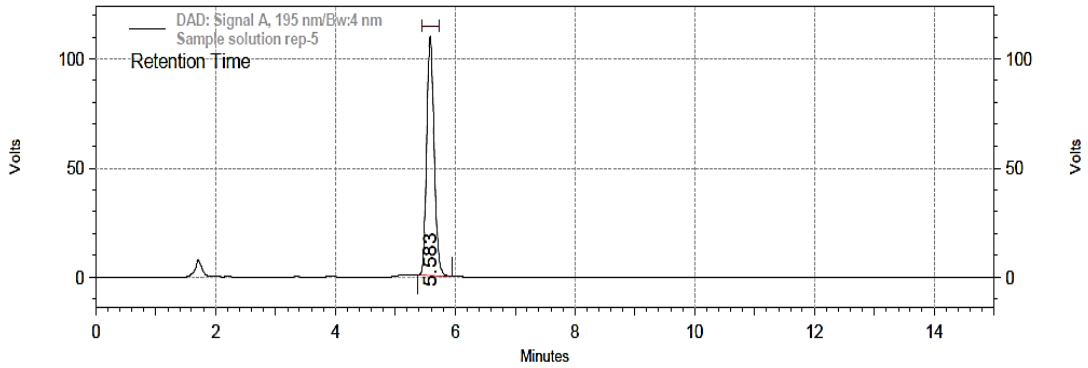


Figure 33: HPLC chromatogram of Cyclophosphamide for repeatability (inj-5)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.583	2048155	9615	1.18

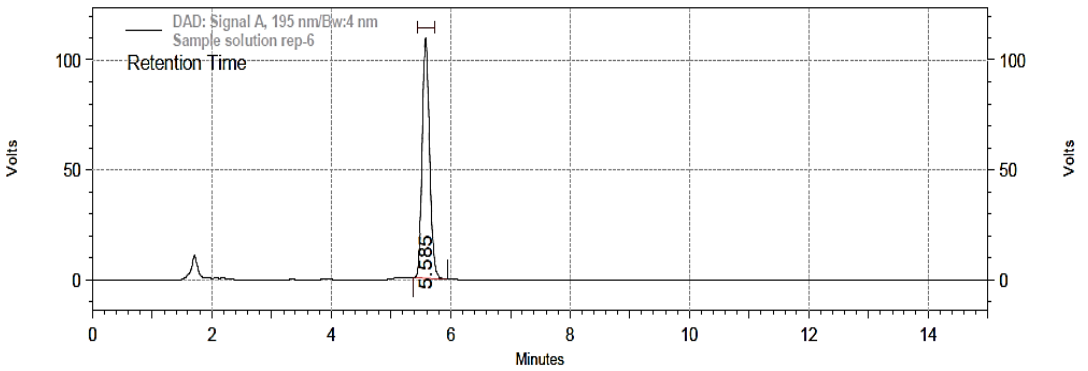


Figure 34: HPLC chromatogram of Cyclophosphamide for repeatability (inj-6)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.585	2051452	9645	1.17

termediate precision

The Intermediate precision studies were studied by six replicate measurements at 0.1mg/mL for Cyclophosphamide.

Table 10: Intermediate Precision results of Cyclophosphamide.

Drug Conc.	**S.No**	**Area**
0.1mg/mL	**Inj-1**	2056122
	Inj-2	2048131
	Inj-3	2045148
	Inj-4	2055172
	Inj-5	2058188
	Inj-6	2061102
	Average	2053977
	STDEV	6109.89
	RSD	**0.29**

Discussion: The % RSD value indicates a good degree of precision within the specified range.

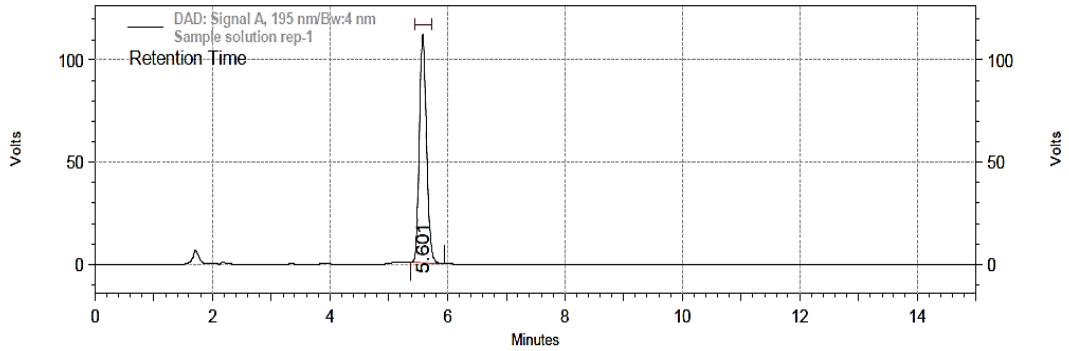


Figure 35: HPLC chromatogram of Cyclophosphamide for intermediate precision (inj-1)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.601	2056122	9029	1.15

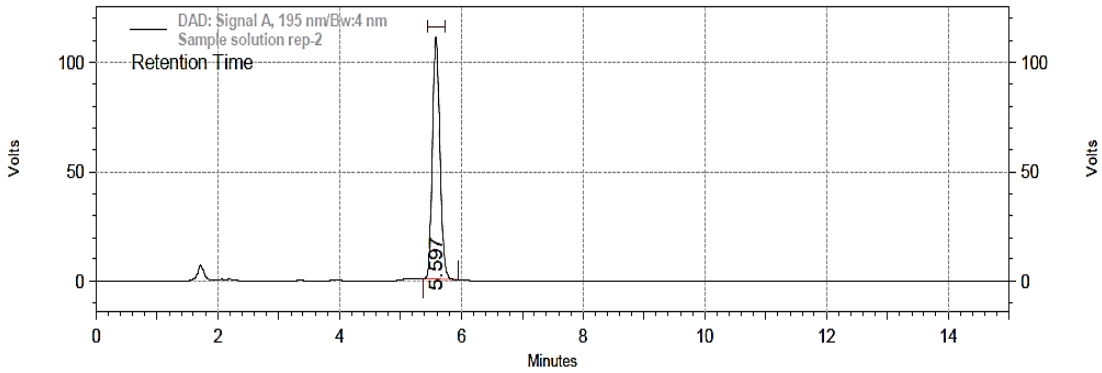


Figure 36: HPLC chromatogram of Cyclophosphamide for intermediate precision (inj-2)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.597	2048131	9231	1.17

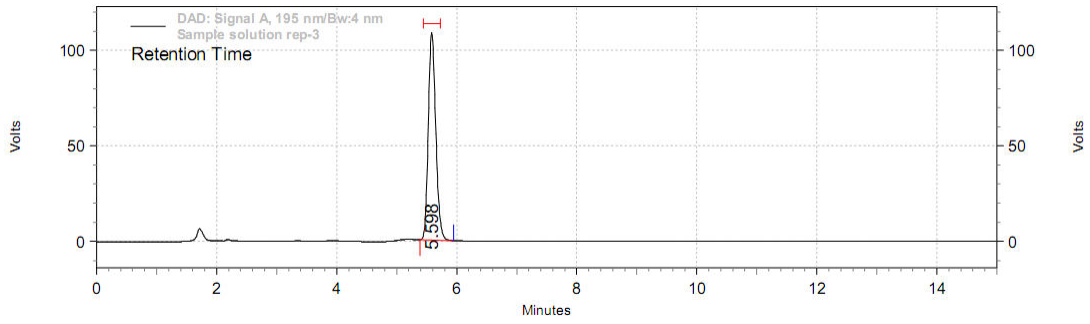


Figure 37: HPLC chromatogram of Cyclophosphamide for intermediate precision (inj-3)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.598	2045148	9437	1.14

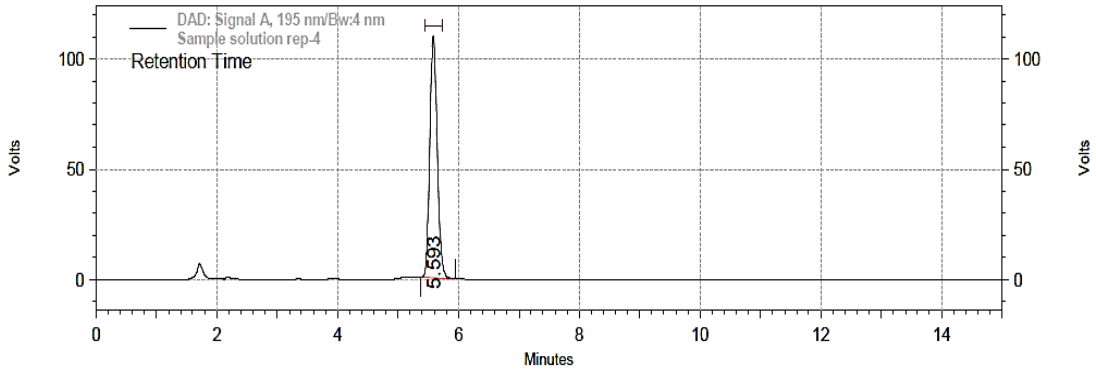


Figure 38: HPLC chromatogram of Cyclophosphamide for intermediate precision (inj-4)

Peak results

Peak	Retention Time	Area	Theoretical plates (USP)	Asymmetry
			9520	
Cyclophosphamide	5.593	2055172		1.11

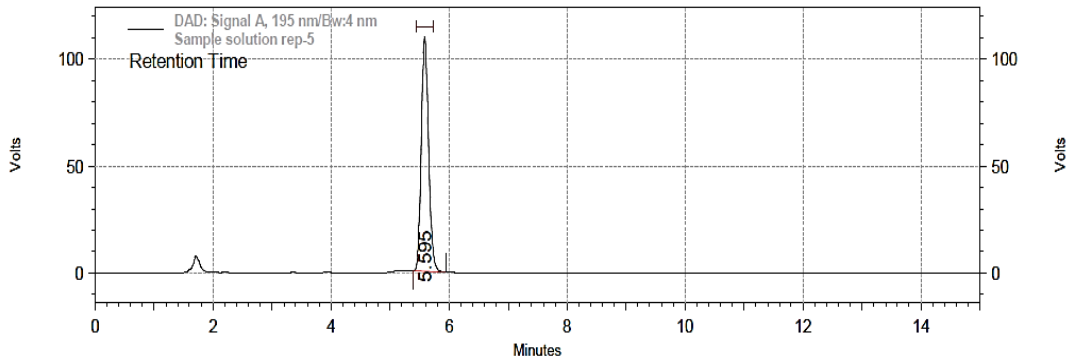


Figure 39: HPLC chromatogram of Cyclophosphamide for intermediate precision (inj-5)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.595	2058188	9643	1.16

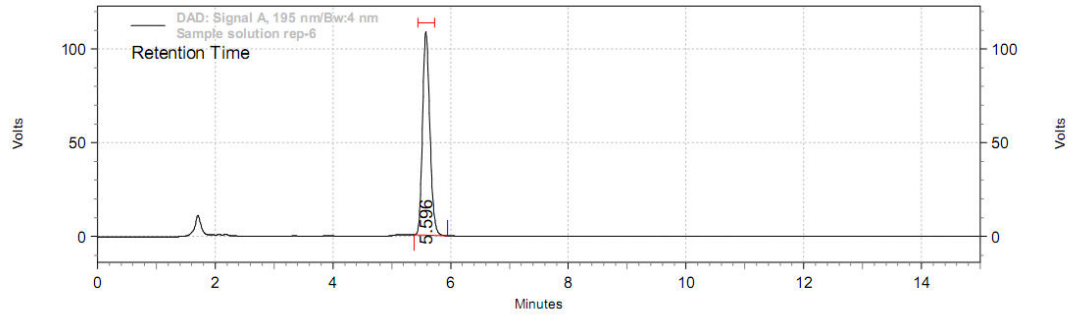


Figure 40: HPLC chromatogram of Cyclophosphamide for intermediate precision (inj-6)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.596	2061102	9633	1.17

Discussion

The %relative standard deviation of Cyclophosphamide for repeatability was found to be 0.21.

The %relative standard deviation of Cyclophosphamide for intermediate precision was found to be 0.29.

Hence the % RSD values indicate a good degree of precision within the specified range.

e) LIMIT OF DETECTION

LOD was calculated by using standard deviation and slope values obtained from calibration curve.

Discussion

The LOD value of Cyclophosphamide was found to be 0.131µg/mL.

f) LIMIT OF QUANTIFICATION

LOQ was calculated by using standard deviation and slope values obtained from calibration curve.

Discussion

The LOQ value of Cyclophosphamide was found to be 0.398µg/mL.

g) ROBUSTNESS

To establish the robustness of the HPLC method employed for analysis of assay of Cyclophosphamide, the method was challenged for various parameters like effect of mobile phase flow, changes in mobile phase composition and change in column. The observations in different conditions are tabulated below:

Table-11: Effect of mobile phase flow (□□20% of Actual flow)

	Actual flow (0.5 ml/min)	**20% Less flow (0.4 ml/min)**	**20% Excess flow (0.6 ml/min)**
Retention time (min)	5.587	5.610	5.577
Theoretical Plates	10383	9075	8878
Tailing Factor	1.12	1.09	1.08

Table-12: Effect of changes in mobile phase composition (□□1% Organic solvent)

	Actual MP	**1% Excess Organic solvent**	**1% Less Organic solvent**
Retention time (min)	5.587	5.450	5.610
Theoretical Plates	10383	8917	6398
Tailing Factor	1.12	1.14	1.15

Table-13: Column to column variation

	LCC11-003	LCC11-002
Retention time (min)	5.587	5.595
Theoretical Plates	10383	9523
Tailing Factor	1.12	1.05

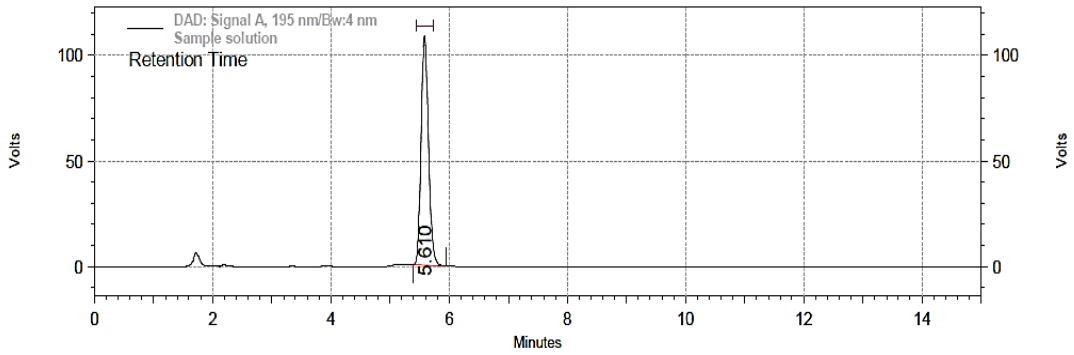


Figure 41: HPLC chromatogram of Cyclophosphamide for robustness (flow 1)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.610	2078518	9075	1.09

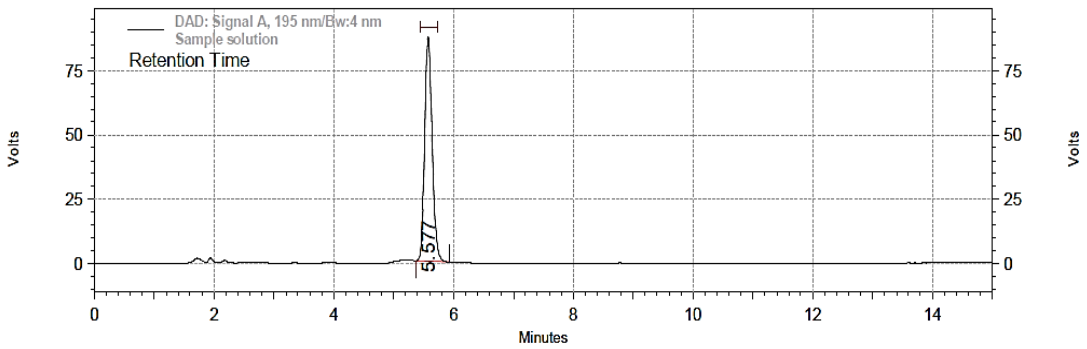


Figure 42: HPLC chromatogram of Cyclophosphamide for robustness (flow 2)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.577	2066717	8878	1.08

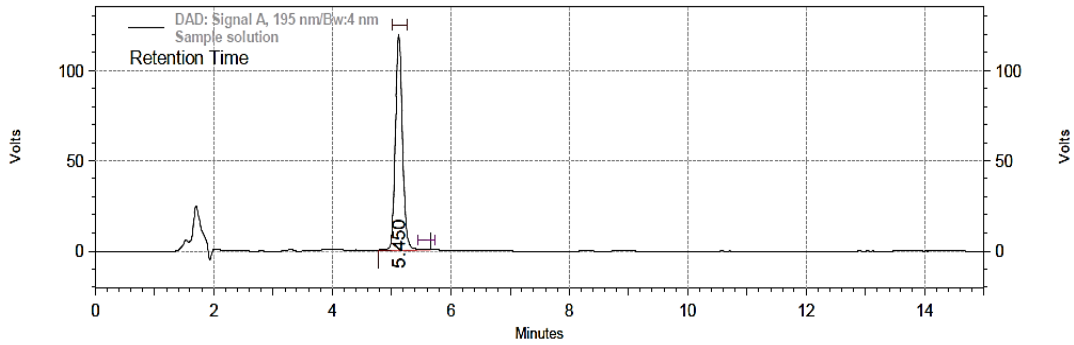


Figure 43: HPLC chromatogram of Cyclophosphamide for robustness (1% excess organic solvent)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamid	5.450	2076737	8917	1.14

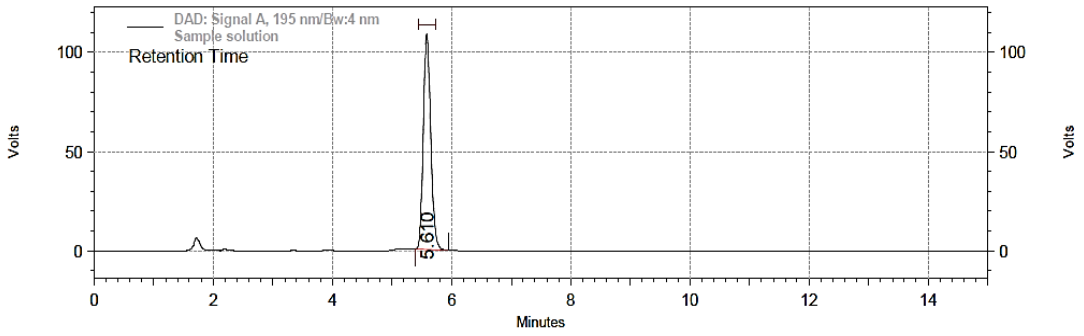


Figure 44: HPLC chromatogram of Cyclophosphamide for robustness (1% less organic solvent)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.610	2008119	6398	1.15

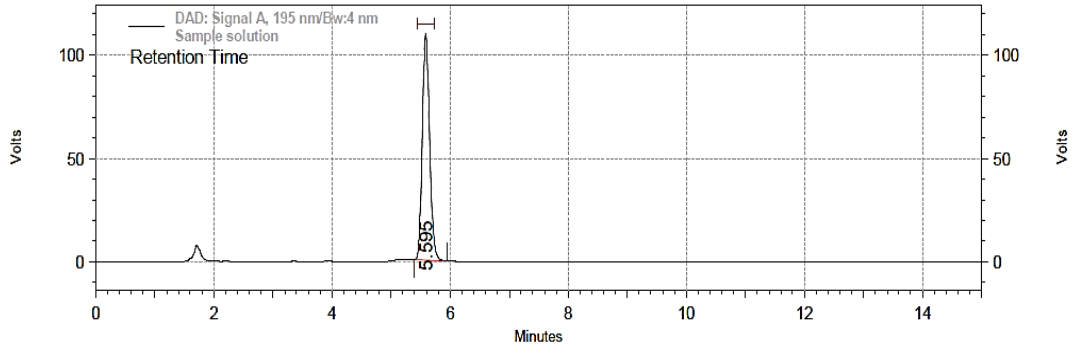


Figure 45: HPLC chromatogram of Cyclophosphamide for robustness (Different column)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.595	2055288	9523	1.05

Discussion:

The robustness was tested by changing the flow rate, Mobile phase composition and column. And it was found that the system suitability parameters were within the acceptance criteria.

h) SYSTEM SUITABILITY

Table 14: system suitability data of Cyclophosphamide

S.no.	System Suitability Parameter	Observations	Proposed Acceptance Criteria
1.	% RSD for Five replicate injections of analyte peak in	0.2	Should be not more than 2.0%
2.	Tailing factor for analyte peak in	1.11	Should be not more
3.	Plate count for analyte peak in standard	9176	Should be not less than

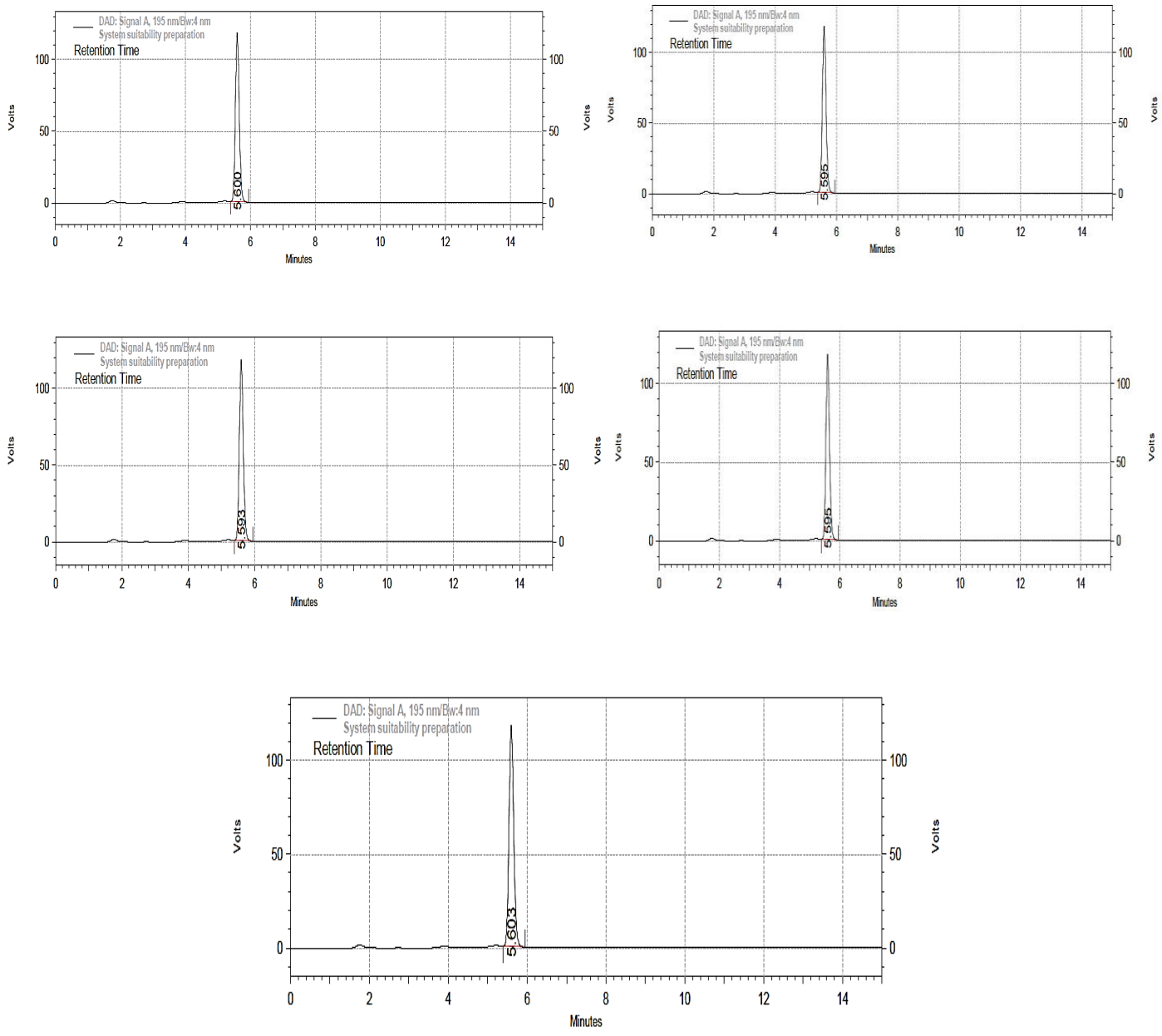


Figure 46: HPLC chromatograms of Cyclophosphamide (system-suitability)

Peak Results of Cyclophosphamide Standard

S.NO	Cyclophosphamide		
	Area	Asymmetry	Plate Count
1	2050439	1.11	10383
2	2051086	1.14	10210
3	2051529	1.17	9848
4	2051650	1.14	9788
5	2051710	1.15	9785
Mean	2051283		
%RSD	0.2		

Discussion

The all system suitability parameters are within the acceptance criteria

7.4. Assay for marketed formulation

Peak results for Standard Solution of Cyclophosphamide (Assay-Development)

Cyclophosphamide				
S.No	**RT (min)**	**Area**	**Plate Count**	**Tailing**
1	5.600	2050439	10383	1.11
2	5.595	2051086	10210	1.14
3	5.593	2051529	9848	1.17
4	5.595	2051650	9788	1.14
5	5.603	2051710	9785	1.15

Table 15: Peak results for Sample Solution of CP (Assay Development)

S.no	Tablet weight in mg	105.5
1	Area(injection-1)	2047489
2	Area(injection-2)	2048570
	Average area	2048029
	Assay	99.5

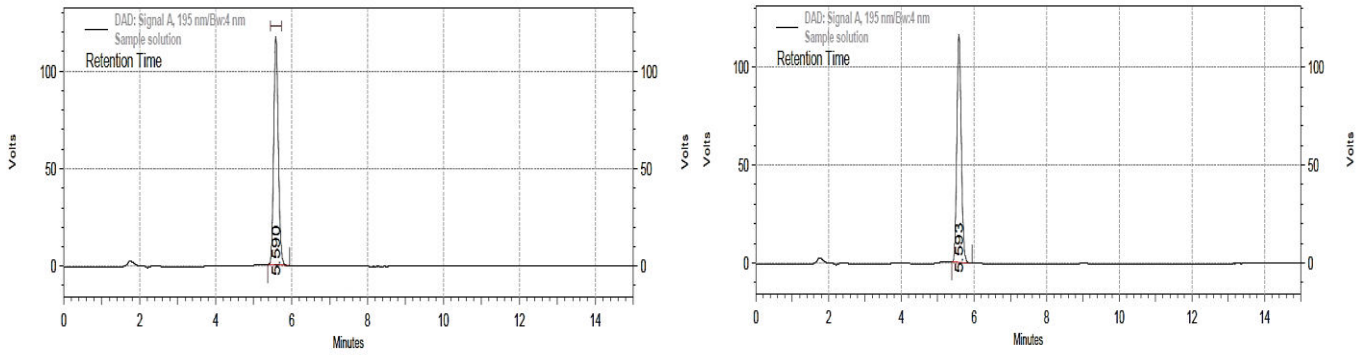


Figure 47: HPLC Chromatograms for sample solutions (Assay-Development)

Peak results

Peak	Retention Time	Area	Theoretical	Asymmetry
Cyclophosphamide	5.590	2047489	10264	1.14

Discussion

The percentage purity of Cyclophosphamide was found to be 99.5. So this method was suitable for analysing the marketed formulations.

8.0. SUMMARY AND CONCLUSION

8.1. SUMMARY

Chapter 1 Opens with introduction giving a brief account of various aspects like chromatography, analytical method development and validation,

Chapter 2 Explains about the drug profile of drug and literature review of various papers regarding about the drug. The chromatographic conditions from the papers were considered for developing new analytical method for Cyclophosphamideby RP-HPLC.

Chapter 3 Explains the aim & objective of present investigation adopted for selected drugs.

Chapter 4 Explains about the plan of the work.

Chapter 5 Contains the materials used for the study

Chapter 6 Containsexperimental investigation regarding theinformation about the chromatographic conditions of various method development trials and procedures for analytical method validation as per ICH guidelines.

Chapter 7 Consists of results obtained and the discussions about the results indicating the acceptance of the results. Tables of the results obtained for validation have been included below

Chapter 8 Contains the summary of the research and conclusion.

Chapter 9 Contains the references form which information of the titled drugs and introduction of general methodology was collected

CONCLUSION

The present research is to developed and validated a precise, accurate and robust method for the estimation of Cyclophosphamide from tablet dosage form and the extensive literature survey carried out revealed that several methods have been reported for simultaneous estimation of Cyclophosphamide i.e., in combination with other drugs. However, there is no method reported for individual estimation in solid dosage form.

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